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Award Number: DAMD17-97-1-7327

TITLE: Characterization of a  $\beta$ -Catenin-Associated Kinase

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REPORT DATE: August 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20020904 033

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 2001	3. REPORT TYPE AND DATES COVERED Final (01 Aug 97 - 31 Jul 01)		
4. TITLE AND SUBTITLE Characterization of a $\beta$ -Catenin-Associated Kinase		5. FUNDING NUMBERS DAMD17-97-1-7327		
6. AUTHOR(S) Stephen Byers Keith Orford				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057  E-Mail: byerss@georgetown.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT ( <i>Maximum 200 Words</i> )  <p><math>\beta</math>-catenin is an important regulator of cell-cell adhesion and embryonic development that associates with and regulates the function of the LEF/TCF family of transcription factors. Mutations of <math>\beta</math>-catenin and the tumor suppressor gene APC occur in human cancers but it is not known if and by what mechanism increased <math>\beta</math>-catenin causes cellular transformation. This study was the first to show a serine phosphorylation-dependent regulation of <math>\beta</math>-catenin ubiquitination and degradation. We went on to demonstrate that modest over-expression of <math>\beta</math>-catenin in a normal epithelial cell results in cellular transformation. These cells form colonies in soft agar, survive in suspension, and continue to proliferate at high cell density and following <math>\gamma</math>-irradiation. Endogenous cytoplasmic <math>\beta</math>-catenin levels and signaling activity were also found to oscillate during the cell cycle. Taken together, these data point to a role of <math>\beta</math>-catenin in the regulation of the G<sub>1</sub> to S phase transition and suspension-induced apoptosis (anoikis). Additional results point to the important role played by two serine kinases (IKK and atypical PKC) in the normal phosphorylation and regulation of <math>\beta</math>-catenin signaling activity.</p>				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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## Introduction

One of the hallmarks of the neoplastic process is the failure of transformed cells to stop dividing when in contact with their neighbors. The mechanism(s) whereby normal cells exhibit contact inhibition and the alterations in these pathways that result in the ability of cancer cells to overcome contact inhibition are poorly understood. Likely candidates for the transmission of the contact/proliferation signal are members of the cadherin family of cell-cell adhesion molecules, and their associated proteins, catenins.

$\beta$ -catenin is also a member of the Wnt signaling pathway, which regulates several developmental pathways. Increases in cytoplasmic  $\beta$ -catenin and  $\beta$ -catenin signaling are also associated with numerous cancers. The oncogenic and developmental effects of  $\beta$ -catenin are mediated by its interaction with and activation of members of the LEF/TCF family of transcription factors. Although much is now known about this signaling system, the actual cellular processes in which  $\beta$ -catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell-cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that  $\beta$ -catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of  $\beta$ -catenin are related and that it may be, at least in part, the mechanistic link between cell-cell adhesion, contact inhibition and/or apoptosis. However, before our work, no studies directly tested the hypothesis that  $\beta$ -catenin is actually oncogenic.

Work carried out in this proposal demonstrate that  $\beta$ -catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition,  $\beta$ -catenin confers resistance to suspension-mediated apoptosis (anoikis) and radiation damage and allows cells to continue cycling when cultured at confluence. In short,  $\beta$ -catenin transforms normal epithelial cells in culture.

The signaling activity of  $\beta$ -catenin is regulated at the level of cytoplasmic protein stability. The wnt signal transduction pathway regulates  $\beta$ -catenin stability by inhibiting the activity of the GSK-3/axin complex that, in the absence of wnt, is able to phosphorylate  $\beta$ -catenin and target it for ubiquitination. The N-terminal of  $\beta$ -catenin contains a region rich in serine and threonine residues that are likely targets for stability regulating kinases. Several of these potential phosphorylation sites are mutated in a number of different cancers strongly pointing to their importance in regulating the transforming activity of  $\beta$ -catenin. Only one of these serines is present within a GSK-3 consensus suggesting that other kinases are also likely to phosphorylate  $\beta$ -catenin and regulate its ubiquitination in a wnt and GSK-3-independent manner. Our results demonstrated that certain PKC inhibitors caused a dramatic accumulation of cytoplasmic  $\beta$ -catenin by inhibiting its ubiquitination. The inhibitor profile indicated that an atypical PKC regulates  $\beta$ -catenin accumulation. In addition, we defined a six amino acid motif that targets both  $\beta$ -catenin and the inhibitor of NF $\kappa$ B, called I $\kappa$ B $\alpha$ , for ubiquitination. A single serine to alanine mutation within this ubiquitination targeting sequence (UTS) stabilized the protein by inhibiting its ubiquitination. Further parallels between  $\beta$ -catenin and I $\kappa$ B $\alpha$  have since been discovered. The ubiquitin ligase complex that recognizes the I $\kappa$ B $\alpha$  and  $\beta$ -catenin UTS contains similar components. The identity of the kinase that directly phosphorylates the serines in the I $\kappa$ B $\alpha$  UTS was recently identified (IKK) and we show in our work that this complex is also involved in the regulation of  $\beta$ -catenin phosphorylation and signaling.

## Methods and Results

The key methodology and results have been included in the annual reports and presented in 4 papers published in J Biol Chem and J Cell Biol. The full text of the papers is included in the appendix.

### Key Research Accomplishments:

1. Demonstration that phosphorylation of  $\beta$ -catenin serine residues 33 and 37 targeted the protein for ubiquitination and subsequent degradation. This work formed the basis for many other studies from other laboratories that elucidated the nature of the SCF complex required for coupling phosphorylated  $\beta$ -catenin (and I $\kappa$ B) to the ubiquitination machinery.
2. The first demonstration that  $\beta$ -catenin was in fact oncogenic and regulated contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest.
3. The first demonstration that a kinase, other than GSK-3, was involved in  $\beta$ -catenin phosphorylation.
4. The first demonstration that significant cross-regulation of  $\beta$ -catenin and NF $\kappa$ B signaling pathways occurs through the I $\kappa$ B kinases.

### Reportable Outcomes:

- Lamberti, C., Lin, K-M., Yamamoto, Y., Verma, IM., Byers, S. and Gaynor, R. (2001) Regulation of  $\beta$ -catenin function by the I $\kappa$ B kinases J Biol Chem. 276:42276-42286.
- Orford, K., Orford, C. and Byers, S. (1999)  $\beta$ -catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. J. Cell Biol. 146: 855-867
- Easwaran, V., Song, V., Polakis, P. and Byers, S. (1999) The ubiquitin-proteasome pathway and serine kinase activity regulate APC modulation of  $\beta$ -catenin/LEF signaling. J. Biol Chem 274: 16641-16645.
- Orford, K., Crockett, C., Weissman, A. and Byers, S.W. 1997. Serine phosphorylation-regulated ubiquitination and proteosomal degradation of  $\beta$ -catenin. J. Biol. Chem. 272: 24735-38

The three papers published before 2001 have already been cited over 100 times in the on-line literature. This translates to an impact factor of over 30/paper, not taking into account citations in reviews and other journals that are not on line. By any measure this is an outstanding citation index and indicates the general importance and impact of the work supported by this grant. This work was solely supported by the DOD for several years and emphasizes the important role of DOD funding

Degrees supported by DAMD17-1-97-7327

Keith Orford MD. PhD 2001

Personnel supported by DAMD17-1-97-7327

Keith Orford MD PhD

### Conclusions:

The wnt/ $\beta$ -catenin and NF $\kappa$ B pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF- $\kappa$ B pathway is involved in the induction of the inflammatory response.  $\beta$ -catenin, a known oncogene, is an important component of the wnt signaling pathway and I $\kappa$ B $\alpha$  is an important regulator of the NF- $\kappa$ B pathway. Both proteins are phosphorylated at

serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of I $\kappa$ B $\alpha$  while GSK-3 $\beta$  is thought to regulate  $\beta$ -catenin phosphorylation. The IKK complex contains two kinases, IKK $\alpha$  and IKK $\beta$ . Gene disruption studies in mice indicate that IKK $\beta$  is the dominant component of the IKK complex involved in phosphorylation of I $\kappa$ B $\alpha$ . We now show that IKK also exists in a complex with  $\beta$ -catenin and that expression of either IKK $\alpha$  or IKK $\beta$  can decrease  $\beta$ -catenin signaling in APC-mutant colon cancer cells with high endogenous  $\beta$ -catenin levels. However, only a dominant negative (DN) IKK $\alpha$  mutant increased  $\beta$ -catenin signaling and protein levels in cells with low endogenous  $\beta$ -catenin. In addition, DN IKK $\alpha$ , but not DN IKK $\beta$ , completely inhibited the ability of APC to decrease  $\beta$ -catenin signaling in colon cancer cells. These results indicate that, in contrast to IKK control of NF $\kappa$ B signaling, IKK $\alpha$  not IKK $\beta$  has the dominant role in the regulation of  $\beta$ -catenin activity.

#### **Appendices (manuscripts and papers not previously submitted)**

Lamberti, C., Lin, K-M., Yamamoto, Y., Verma, IM., Byers, S. and Gaynor, R. (2001) Regulation of  $\beta$ -catenin function by the I $\kappa$ B kinases J Biol Chem. 276:42276-42286.

## Regulation of $\beta$ -Catenin Function by the I $\kappa$ B Kinases\*

Received for publication, May 10, 2001, and in revised form, August 28, 2001  
Published, JBC Papers in Press, August 29, 2001, DOI 10.1074/jbc.M104227200

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Both the  $\beta$ -catenin and the nuclear factor  $\kappa$ B (NF- $\kappa$ B) proteins are important regulators of gene expression and cellular proliferation. Two kinases, IKK $\alpha$  and IKK $\beta$ , are critical activators of the NF- $\kappa$ B pathway. Here we present evidence that these kinases are also important in the regulation of  $\beta$ -catenin function. IKK $\alpha$ - and IKK $\beta$ -deficient mouse embryo fibroblasts exhibited different patterns of  $\beta$ -catenin cellular localization. IKK $\beta$  decreases  $\beta$ -catenin-dependent transcriptional activation, while IKK $\alpha$  increases  $\beta$ -catenin-dependent transcriptional activity. IKK $\alpha$  and IKK $\beta$  interact with and phosphorylate  $\beta$ -catenin using both *in vitro* and *in vivo* assays. Our results suggest that differential interactions of  $\beta$ -catenin with IKK $\alpha$  and IKK $\beta$  may in part be responsible for regulating  $\beta$ -catenin protein levels and cellular localization and integrating signaling events between the NF- $\kappa$ B and Wntless pathways.

$\beta$ -Catenin, the mammalian homologue of the *Drosophila armadillo* protein, is a ubiquitously expressed protein that has at least two distinct roles in the cell. First, it participates in cell-cell adhesion by mediating the association of E-cadherin with the cytoskeleton (1, 2). Second, it is a critical downstream component of the Wnt<sup>1</sup> or Wntless signal transduction pathway (3–5). The Wnt family of secretory glycoproteins plays an important role in embryonic development, in the induction of cell polarity, and in the determination of cell fate. Deregulation of Wnt signaling disrupts axis formation in embryos (5–8) and is associated with multiple human malignancies (9).

The current model of Wnt signaling indicates that the binding of the Wnt proteins to their receptor, frizzled, stabilizes  $\beta$ -catenin by inhibiting the activity of a serine/threonine kinase glycogen synthase kinase-3 or GSK-3 $\beta$  (9). GSK-3 $\beta$  is associated with  $\beta$ -catenin in a multiprotein complex that includes the adenomatous polyposis coli tumor suppressor protein (APC),

axin or conductin, protein phosphatase 2A, and dishevelled. GSK-3 $\beta$  phosphorylation of residues in the amino terminus of  $\beta$ -catenin results in APC-mediated  $\beta$ -catenin degradation via the ubiquitin-proteasome pathway (10, 11). Increased levels of  $\beta$ -catenin are frequently found in colon cancer due to mutations in either the APC gene (12–14) or at residues in the amino terminus of  $\beta$ -catenin that are phosphorylated by GSK-3 $\beta$  (15–17). In the nucleus,  $\beta$ -catenin forms a complex with members of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) family and activates gene expression of a variety of target genes (18–23) including *c-myc* (24) and cyclin D1 (25, 26).

NF- $\kappa$ B comprises a family of transcription factors which are critical in activating the expression of genes involved in the immune and inflammatory response and in the regulation of cellular apoptosis (27, 28). NF- $\kappa$ B is sequestered in the cytoplasm by a family of inhibitory proteins known as I $\kappa$ B. Upon stimulation of this pathway by a variety of agents including IL-1 and TNF $\alpha$ , the kinases IKK $\alpha$  and IKK $\beta$  (29–33) in conjunction with the scaffold protein IKK $\gamma$ /NEMO (34–36) leads to the phosphorylation of I $\kappa$ B $\alpha$  at serine residues 32 and 36. Gene disruption studies in mice indicate that IKK $\beta$  appears to be the critical kinase in activating the NF- $\kappa$ B pathway (37–39), while IKK $\alpha$  appears to be critical for other functions such as keratinocyte differentiation (40–42). IKK $\alpha$  and IKK $\beta$  can form homodimers and also heterodimerize with each other, and this process is critical for their kinase activity. IKK phosphorylation of I $\kappa$ B $\alpha$  leads to its ubiquitination and degradation by the 26S proteasome and the nuclear translocation of NF- $\kappa$ B (43).

Interestingly, the sequence DSGXXS, which is the target site in I $\kappa$ B for IKK phosphorylation, is also found in the amino terminus of  $\beta$ -catenin (11). Phosphorylation of this sequence in both  $\beta$ -catenin and I $\kappa$ B stimulates their interactions with the ubiquitin ligase  $\beta$ -TrCP leading to their degradation by the proteasome (10, 11, 44). It has also been demonstrated that the  $\beta$ -catenin/TCF complex increases  $\beta$ -TrCP levels by a post-transcriptional mechanism to result in opposite effects on  $\beta$ -catenin and NF- $\kappa$ B activity (45). In addition, disruption of either the murine GSK-3 $\beta$  and IKK $\beta$  genes result in a similar phenotype with embryonic lethality due to hepatic apoptosis from increased sensitivity to TNF $\alpha$  (46). These results suggest potential relationships between  $\beta$ -catenin and NF- $\kappa$ B signaling pathways.

Given the fact that both the NF- $\kappa$ B and  $\beta$ -catenin pathways are important in the control of cellular proliferation and are regulated by cellular kinases that lead to  $\beta$ -TrCP-mediated degradation (10, 11, 45), we explored potential similarities in their regulation. First, we addressed whether there were differences in the cellular localization of  $\beta$ -catenin in wild-type mouse embryo fibroblasts as compared with fibroblasts derived from IKK $\alpha$ - and IKK $\beta$ -deficient mice. Next, we analyzed inter-

\* This work was supported by National Institutes of Health Grant CA 74128. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: Wnt, Wntless; APC, adenomatous polyposis coli tumor suppressor protein; TCF, T-cell factor; LEF, lymphocyte-enhancer factor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; IKK, I $\kappa$ B kinase; MEF, mouse embryo fibroblast; FITC, fluorescein isothiocyanate; NIK, NF- $\kappa$ B inducing kinase; GST, glutathione S-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

actions between both IKK $\alpha$  and IKK $\beta$  and  $\beta$ -catenin and determined whether these kinases regulated  $\beta$ -catenin-dependent transcriptional activity. The results of this analysis indicate that IKK $\alpha$  can positively regulate  $\beta$ -catenin-dependent transcriptional activity while IKK $\beta$  negatively regulates this activity.

#### MATERIALS AND METHODS

**Cells and Reagents**—SW480 cells were purchased from American Tissue Culture Collection (Manassas, VA) and maintained in L-15 medium supplemented with 10% fetal bovine serum (HyClone Laboratories), 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). COS, mouse embryo fibroblasts (MEFs, a kind gift of Xiaodong Wong), IKK $\alpha$  and IKK $\beta$  knock-out cells (39, 42) were maintained in Dulbecco's modified Eagle's medium and supplemented with the same components as above.

**Antibodies**—Polyclonal antibodies to IKK $\alpha$  (sc-7182), IKK $\beta$  (sc-7607), and  $\beta$ -catenin (sc-1496) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against  $\beta$ -catenin and TFIIB (Transduction Laboratory), IKK $\alpha$  (PharMingen), the hemagglutinin epitope HA/12CA5 (Roche Molecular Biochemicals), and the FLAG-epitope-M2 (Sigma) were also used in immunoprecipitation and Western blot analysis. Donkey anti-rabbit, anti-mouse and anti-goat antibodies conjugated with either anti-FITC or Red-X rhodamine were obtained from Jackson Laboratory.

**Plasmid Constructs**—The pCMV5 expression plasmids containing either FLAG-tagged IKK $\alpha$  and IKK $\beta$  including the constitutively active kinases (SS/EE) with substitutions at residues 176/180 for IKK $\alpha$  or 177/181 for IKK $\beta$  and the kinase defective (K/M) mutants at residue 44 in both IKK $\alpha$  and IKK $\beta$  were described previously (30, 47, 48). Wild-type and mutant IKK $\alpha$  and IKK $\beta$  cDNAs were each cloned into the baculovirus expression vector pAHLT. The recombinant baculoviruses were used to infect SF9 cells to produce recombinant IKK proteins for *in vitro* kinase assays (48). The pCMV5 expression vectors containing the wild-type and the dominant negative NIK mutant in which lysine residues at positions 429 and 430 were substituted with alanine contained an amino-terminal Myc-tag (48).

The pCMV5 expression vectors encoding full-length human  $\beta$ -catenin was provided by S. Byers, while the plasmids for LEF-1, TOPFLASH, and FOPFLASH were gifts of K. Kinzler and R. Grosschedl. The RSV- $\beta$ -galactosidase construct was a gift from P. Chaudhary. The glutathione S-transferase (GST) full-length  $\beta$ -catenin (GST  $\beta$ -cat-(1-781)) bacterial expression vector was constructed by using polymerase chain reaction (PCR) to generate a fragment encompassing the full-length  $\beta$ -catenin, which was then cloned in frame with GST in the pGEX. The GST fusion protein containing the amino-terminal 91 amino acids of  $\beta$ -catenin was constructed by *Sac*I digestion and ligation of the GST fusion containing wild-type  $\beta$ -catenin. The constructs GST- $\beta$ -cat-(130-781), GST- $\beta$ -cat-(1-400), GST- $\beta$ -cat-(130-400), and GST- $\beta$ -cat-(618-781) were constructed using PCR. The amino-terminal deleted form of  $\beta$ -catenin utilized PCR primers to generate a fragment containing amino acids 130-781, which was cloned into pCMV5 and contained a carboxyl-terminal HA-epitope. All constructs that were generated by PCR were subjected to DNA sequencing and cloned into pcDNA3.

**Expression and Purification of GST  $\beta$ -Catenin Fusion Proteins**—Recombinant GST  $\beta$ -catenin fusion proteins were expressed in bacterial strain BL21 and lysed in HMK buffer (50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride), and the bacterial lysates were incubated with 0.5 ml of packed glutathione-conjugated-agarose beads (Sigma) for 2 h at 4 °C. After three washes, the GST fusion proteins were eluted with 10 mM glutathione in HMK buffer and dialyzed, and protein purity was assessed by SDS-polyacrylamide gel electrophoresis.

**Luciferase Reporter Assays**—COS cells and mouse embryo fibroblasts were plated at 50% confluence in 35-mm tissue culture wells. After 24 h, the cells were transfected using LipofectAMINE Plus with the indicated DNA constructs and either the TOPFLASH luciferase reporter containing LEF/TCF binding sites or the FOPFLASH luciferase reporter with mutated LEF/TCF sites. An NF- $\kappa$ B luciferase reporter containing three NF- $\kappa$ B binding sites upstream of a thymidine kinase minimal promoter was used to detect NF- $\kappa$ B-directed gene expression. An RSV- $\beta$ -galactosidase expression vector was included in the transfection assays to control for differences in transfection efficiency, and the pCMV5 plasmid was added to the transfection assays to standardize DNA quantities. Between 18 to 24 h posttransfection, the cells were washed twice with cold PBS, and the reporter activity was measured using the luciferase assay system (Promega). All transfections were done in duplicate and repeated at least three times.

**Fractionation of Cellular Extracts**—Cytoplasmic extracts were prepared from 10<sup>8</sup> SW480 or COS cells as described previously (49) with slight modifications. Cells were washed twice with cold PBS, and cell pellets were resuspended in 5 volumes of buffer A (10 mM Hepes (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM  $\beta$ -glycerophosphate, 0.5  $\mu$ M okadaic acid, 1 mM sodium orthovanadate), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed with 15 strokes of a Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants termed S100 were collected, mixed with 0.11 volume of buffer B (0.3 M Hepes (pH 7.9), 30 mM MgCl<sub>2</sub>, and 1.4 M NaCl), and then centrifuged at 100,000  $\times g$  for 60 min at 4 °C.

Whole cell extracts were prepared from COS cells transfected with hemagglutinin-tagged  $\beta$ -catenin alone or  $\beta$ -catenin and FLAG-tagged IKK $\alpha$  and IKK $\beta$  as described (47) in lysis buffer containing 40 mM Tris, (pH 8), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 5 mM  $\beta$ -glycerophosphate, 5 mM NaF, 1 mM NaVO<sub>4</sub> (pH 10.0), and protease inhibitors (Roche Molecular Biochemicals).

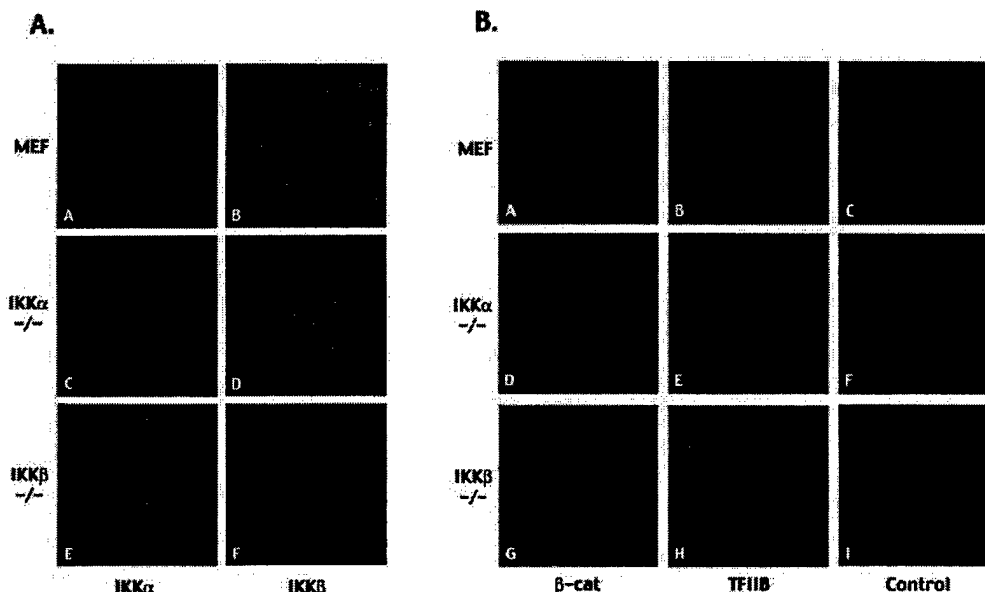
**Gel Filtration Chromatography**—S100 extracts prepared from the SW480 and COS cells were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) and equilibrated with buffer D (20 mM Hepes (pH 7.9), 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column included bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa),  $\beta$ -amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa).

**Protein Association and Western Blot Analysis**—For endogenous protein association studies, equal volumes of proteins (200–300  $\mu$ l) from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1  $\mu$ g of indicated antibodies or normal mouse IgG followed by the addition of protein G-agarose (Sigma) for 2–3 h at 4 °C. For protein association studies using transfected IKK and  $\beta$ -catenin expression vectors, COS cells were transfected with FLAG-tagged IKK $\alpha$  or IKK $\beta$  and HA-tagged  $\beta$ -catenin cDNAs. Cells were harvested 18–24 h after transfection, extracts were prepared, and gel chromatography was performed as described above. Equal volumes of each column fraction were immunoprecipitated with 12CA5 antibody or anti-FLAG M2 antibody. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech), and probed with specific antibodies. The membrane-bound immune complexes were analyzed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). For *in vitro* association studies, 40  $\mu$ l of the cytoplasmic fractions were incubated overnight at 4 °C with 40  $\mu$ l of the glutathione-conjugated-agarose bound with indicated proteins. Following three washes with 10 volumes of cold PBS, the protein complexes were subjected to Western blot analysis as described above.

**In Vitro Kinase Assays**—Kinase assays were performed as described by Yamamoto *et al.* (48). The baculovirus-produced IKK proteins were purified by nickel-agarose chromatography and then immunoprecipitated with 12CA5 monoclonal antibody (48). The epitope-tagged IKK $\alpha$  and IKK $\beta$  kinases were transfected into COS cells, and extracts were immunoprecipitated with the M2 monoclonal antibody directed against the FLAG-epitope. These kinases were added to kinase buffer containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, 1 mM ATP, 1 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and then 1  $\mu$ g of each of the substrates including wild-type or the S32A/S36A of GST-I $\kappa$ B $\alpha$ -(1–54) or GST- $\beta$ -cat-(1–91) were incubated for 15 min at 30 °C. For determination of phosphate incorporation into the GST-I $\kappa$ B $\alpha$ -(1–54) and GST- $\beta$ -catenin-(1–91), 2  $\mu$ g of each of these substrates was incubated with either FLAG-tagged IKK $\alpha$  or IKK $\beta$ , which were immunoprecipitated from COS cell extract with the M2 monoclonal antibody in kinase buffer containing 15  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP with a specific activity of 6000 Ci/mM (New England Nuclear) and either 0.01 mM, 0.01 mM, or 1.0 mM of cold ATP. The kinase reaction mixtures were subjected to electrophoresis on 10% SDS-polyacrylamide gels and autoradiography. The <sup>32</sup>P-labeled I $\kappa$ B $\alpha$  and  $\beta$ -catenin substrates were then subjected to scintillation counting, and the moles of phosphate incorporated were calculated. Reactions were incubated at 30 °C for 5, 15, 30, 60, and 120 min and stopped by the addition of protein loading buffer and heating to 90 °C.

**Immunocytochemistry and Confocal Microscopy**—Cells were cultured overnight on coverslips in Dulbecco's modified Eagle's medium without serum, washed two times with PBS, and fixed with 3.7% formaldehyde for 10 min followed by a brief permeabilization with 0.5% Triton X-100 in PBS. The cells were blocked for 30 min with 3% normal





**FIG. 1. Characterization of  $\beta$ -catenin localization in  $IKK\alpha$ - and  $IKK\beta$ -deficient cells.** A, MEF (panels A and B) and either  $IKK\alpha$ -deficient ( $IKK\alpha^{-/-}$ ) (panels C and D) or  $IKK\beta$ -deficient ( $IKK\beta^{-/-}$ ) (panels E and F) embryo fibroblasts were plated overnight on coverslips before staining with either rabbit polyclonal antibodies directed against  $IKK\alpha$  (panels A, C, and E) or  $IKK\beta$  (panels B, D, and F) followed by staining with a secondary Red-X rhodamine-conjugated rabbit antibody. B, alternatively MEF (panels A–C),  $IKK\alpha^{-/-}$  (panels D–F) and  $IKK\beta^{-/-}$  (panels G–I) cells were stained with either a goat antibody to  $\beta$ -catenin (green) (panels A, D, and G) or a mouse monoclonal antibody to TFIIB (red) (panels B, E, and H). Donkey anti-goat antibody conjugated with FITC and anti-mouse conjugated with Red-X rhodamine were used as secondary antibodies. The respective negative controls utilizing the TFIIB monoclonal antibody and the donkey anti-goat FITC-conjugated antibody are also shown (panels C, F, and I). Images were collected using a laser scanning confocal microscope (Bio-Rad).

donkey serum in PBS and then incubated for 1 h with primary antibodies (diluted 1:50 to 1:200 in 1% normal donkey serum in PBS). The coverslips were washed three times with PBS and then incubated for 1 h with the secondary antibodies conjugated with FITC or Red-X rhodamine (diluted 1:400 in 1% normal donkey serum in PBS). Samples were washed three times and then treated with Aquamount (Polysciences). The results were analyzed on a laser scanning confocal microscope MRC 1000 (Bio-Rad).

#### RESULTS

**$\beta$ -Catenin Localization in  $IKK$ -deficient Cells**—First, the localization of  $IKK\alpha$  and  $IKK\beta$  in wild-type MEFs was compared with that seen in  $IKK\alpha$ -deficient ( $IKK\alpha^{-/-}$ ) and  $IKK\beta$ -deficient ( $IKK\beta^{-/-}$ ) cells using immunofluorescence analysis with confocal microscopy. Wild-type mouse embryo fibroblasts (Fig. 1A, panels A and B),  $IKK\alpha^{-/-}$  (Fig. 1A, C and D), and  $IKK\beta^{-/-}$  (Fig. 1A, panels E and F) cells were plated on coverslips overnight and stained with rabbit polyclonal antibodies directed against either  $IKK\alpha$  or  $IKK\beta$ . In MEFs,  $IKK\alpha$  localized in both the nucleus and the cytoplasm, while  $IKK\beta$  localized predominantly in the cytoplasm (Fig. 1A, panels A and B). In  $IKK\alpha^{-/-}$  cells,  $IKK\beta$  localized predominantly in the cytoplasm (Fig. 1A, panel D). In  $IKK\beta^{-/-}$  cells, there appeared to be increased  $IKK\alpha$  present in the nucleus as compared with that seen in MEFs (Fig. 1A, panel E). There was no  $IKK\alpha$  staining observed in  $IKK\alpha^{-/-}$  cells (Fig. 1A, panel C) or  $IKK\beta$  staining seen in  $IKK\beta^{-/-}$  (Fig. 1A, panel F), thus confirming the identity of these cells.

Immunostaining of MEFs,  $IKK\alpha$ -, and  $IKK\beta$ -deficient cells with a polyclonal antibody directed against  $\beta$ -catenin demonstrated that  $\beta$ -catenin has a different pattern of staining in  $IKK\alpha^{-/-}$  and  $IKK\beta^{-/-}$  cells.  $\beta$ -Catenin was present in both the nucleus and the cytoplasm of MEFs with marked accumulation at cell-cell junctions (Fig. 1B, panel A). In  $IKK\alpha^{-/-}$  cells, there was reduced nuclear staining of  $\beta$ -catenin as compared with MEF cells (Fig. 1B, panel G). There was more  $\beta$ -catenin present in the nucleus and the perinuclear region of  $IKK\beta^{-/-}$  cells than in  $IKK\alpha^{-/-}$  cells (Fig. 1B, panel G). As a control, these cells

were also stained with a monoclonal antibody directed against the basal transcription factor TFIIB, which is localized predominantly in the nucleus (Fig. 1B, panels B, E, and H). There was little background staining when the FITC-conjugated anti-goat secondary antibody was used with the mouse monoclonal antibody directed against TFIIB (Fig. 1B, panels C, F, and I). These results indicate that there is less  $\beta$ -catenin localized in the nucleus of  $IKK\alpha^{-/-}$  cells than in either  $IKK\beta^{-/-}$  cells or MEF cells.

**$\beta$ -Catenin Activity in  $IKK$ -deficient Cells**—Next we addressed whether the differences in  $\beta$ -catenin distribution in the  $IKK$ -deficient embryo fibroblasts could alter its transcriptional activity. The  $IKK$ -deficient cells and the parental MEFs were transfected with a TOPFLASH reporter construct alone or with expression vectors encoding either LEF-1 or  $\beta$ -catenin. The TOPFLASH reporter is driven by four LEF/TCF binding motifs inserted upstream of a minimal c-fos promoter and a luciferase gene (15). As a control, the FOPFLASH reporter, which lacks LEF/TCF binding sites, was utilized. An RSV- $\beta$ -galactosidase expression vector was included in these transfections to control for differences in transfection efficiency.

When the TOPFLASH reporter alone was transfected into  $IKK\alpha^{-/-}$  cells, there was consistently a 5–6-fold lower level of activity as compared with that observed in  $IKK\beta^{-/-}$  cells (Fig. 2). Transfection of an expression vector encoding LEF-1 into either  $IKK\alpha^{-/-}$  or  $IKK\beta^{-/-}$  cells markedly stimulated TOPFLASH activity as did transfection of expression vectors encoding both LEF-1 and  $\beta$ -catenin. There was no significant activity from the FOPFLASH reporter in either the absence or presence of  $\beta$ -catenin and LEF-1 (Fig. 2). Transfection of expression vectors encoding wild-type  $IKK\beta$  and LEF-1 into  $IKK\alpha^{-/-}$  cells reduced TOPFLASH activity, while transfection of an  $IKK\alpha$  expression vector with LEF-1 increased TOPFLASH activity in these cells (Fig. 2). When similar studies were performed in  $IKK\beta^{-/-}$  cells, transfection of an  $IKK\beta$  expression vector reduced TOPFLASH activity, while transfection of an  $IKK\alpha$  ex-

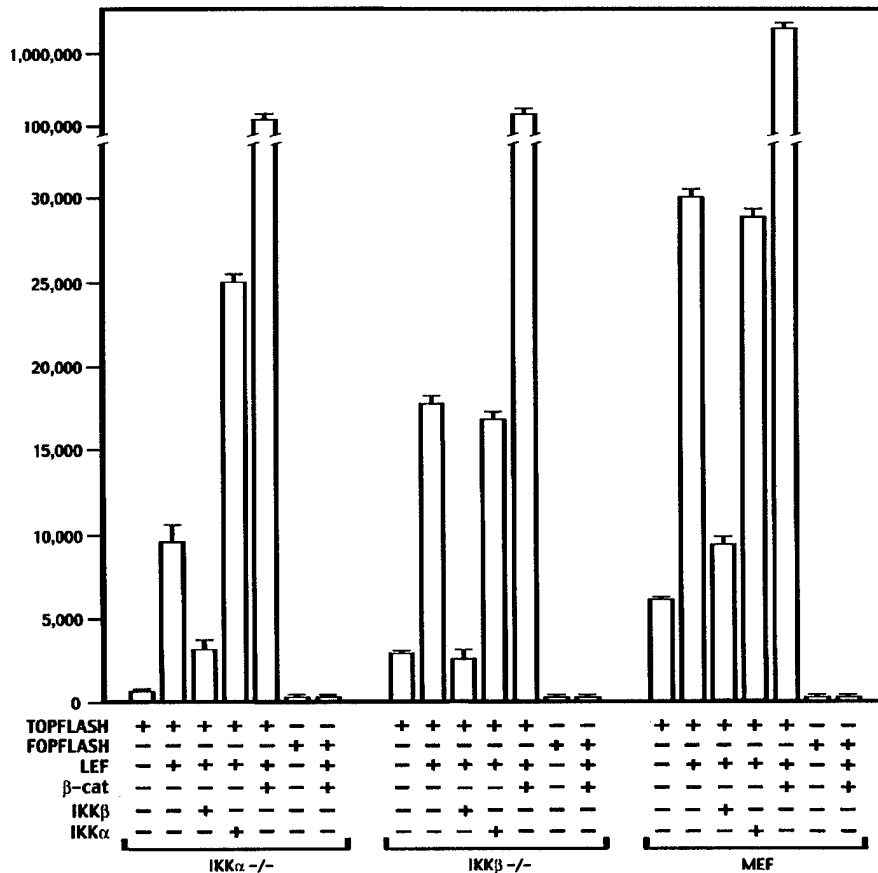


FIG. 2.  $\beta$ -Catenin-mediated gene expression in  $IKK\alpha$ - and  $IKK\beta$ -deficient cells.  $IKK\alpha$ <sup>-/-</sup>,  $IKK\beta$ <sup>-/-</sup>, and MEFs were each cotransfected with either a TOPFLASH or FOPFLASH reporter (0.85  $\mu$ g) and pCMV5 expression vectors encoding  $\beta$ -catenin (0.5  $\mu$ g), LEF-1 (50 ng), and either  $IKK\alpha$  or  $IKK\beta$  (0.5  $\mu$ g) as indicated and an RSV- $\beta$ -galactosidase reporter (0.60  $\mu$ g) using LipofectAMINE Plus (Life Technologies, Inc.). All transfections contained 2.5  $\mu$ g of DNA with a pCMV5 expression vector added to standardize DNA quantities. After 18 h, the cells were collected and lysed, both luciferase and  $\beta$ -galactosidase activity was determined, and the normalized luciferase activity was calculated by correcting for differences in  $\beta$ -galactosidase activity.

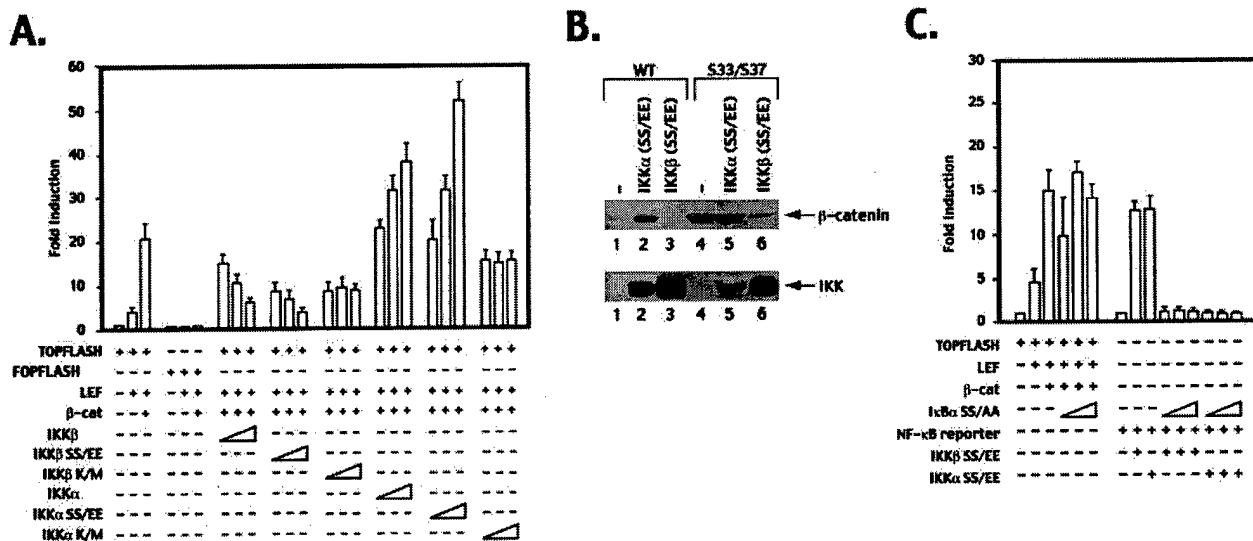
pression vector did not significantly alter TOPFLASH activity (Fig. 2). Transfection of both  $\beta$ -catenin and LEF-1 resulted in similar levels of TOPFLASH activity in the  $IKK\beta$ <sup>-/-</sup> and  $IKK\alpha$ <sup>-/-</sup> cells (Fig. 2). The parental MEF cells consistently gave somewhat higher TOPFLASH activity than seen in the  $IKK$ -deficient cells (Fig. 2). Again transfection of an  $IKK\beta$  expression vector with LEF-1 into these cells reduced TOPFLASH activity, while transfection of an  $IKK\alpha$  expression vector with LEF-1 resulted in little change in TOPFLASH activity (Fig. 2). Thus, the reduced levels of endogenous  $\beta$ -catenin in the nuclei of  $IKK\alpha$ <sup>-/-</sup> cells are associated with decreased  $\beta$ -catenin activation of gene expression, and this defect could be complemented by transfection of an  $IKK\alpha$  expression vector.  $IKK\alpha$  does not increase gene expression in the  $IKK\beta$ <sup>-/-</sup> and MEF cells, which have relatively abundant levels of nuclear  $\beta$ -catenin.

***IKK $\beta$  and  $IKK\alpha$  Have Differential Effects on  $\beta$ -Catenin Transactivation***—The results presented in the previous section suggested that  $IKK\beta$  and  $IKK\alpha$  could potentially be involved in regulating the transcriptional stimulatory properties of  $\beta$ -catenin. Thus it was important to address whether either  $IKK\alpha$  or  $IKK\beta$  could alter  $\beta$ -catenin-mediated transcriptional activation in COS cells, which have low levels of endogenous  $\beta$ -catenin in the nucleus and relatively low levels of  $IKK\alpha$  and  $IKK\beta$  (data not shown). COS cells were transfected with either a TOPFLASH or FOPFLASH reporter, LEF-1 and  $\beta$ -catenin expression vectors, and increasing amounts of expression vectors

encoding either the wild-type, constitutively active or kinase-defective mutants of  $IKK\alpha$  and  $IKK\beta$ . The constitutively active  $IKK$  proteins ( $IKK\alpha$  SS/EE and  $IKK\beta$  SS/EE) have glutamate substituted for serine residues in their T-loop so as to mimic phosphorylation of these residues and increase the activity of these kinases (30). The kinase-defective mutants ( $IKK\alpha$  K/M and  $IKK\beta$  K/M) contain a substitution of a lysine residue at position 44 with methionine (30).

As previously demonstrated, the coexpression of  $\beta$ -catenin and LEF-1 increased TOPFLASH but not FOPFLASH activity (Fig. 3A). When either wild-type  $IKK\beta$  or the constitutively activate kinase,  $IKK\beta$  SS/EE, was cotransfected with  $\beta$ -catenin and LEF-1, TOPFLASH activity decreased in a concentration-dependent manner (Fig. 3A). In contrast, cotransfection of either wild-type  $IKK\alpha$  or the constitutively active kinase,  $IKK\alpha$  SS/EE, increased  $\beta$ -catenin-dependent transactivation in a concentration-dependent manner (Fig. 3A). Transfection of the  $IKK\beta$  K/M mutant resulted in a modest decrease in  $\beta$ -catenin transactivation that was not concentration-dependent, while transfection of the  $IKK\alpha$  K/M mutant did not significantly alter  $\beta$ -catenin transactivation (Fig. 3A).

The cotransfection experiments in COS cells indicated that  $IKK\alpha$  increased  $\beta$ -catenin-dependent gene expression, while  $IKK\beta$  decreased  $\beta$ -catenin-dependent gene expression. Thus, we investigated whether  $IKK\alpha$  and  $IKK\beta$  can alter  $\beta$ -catenin protein levels. In addition, we asked whether  $IKK\alpha$  and  $IKK\beta$



**FIG. 3. Role of IKK $\alpha$  and IKK $\beta$  in  $\beta$ -catenin transactivation.** A, the TOPFLASH reporter (0.75  $\mu$ g) and expression vectors encoding LEF-1 (50 ng) and  $\beta$ -catenin (0.7  $\mu$ g) were cotransfected into COS cells in the presence of wild-type IKK $\alpha$  or IKK $\beta$ , the constitutively active kinases, IKK $\beta$  SS/EE and IKK $\alpha$  SS/EE, or the kinase-defective mutants, IKK $\beta$  K/M and IKK $\alpha$  K/M, utilizing either 0.2, 0.5 or 1.0  $\mu$ g of these kinases. B, COS cells were transfected with pCMV5 expression vectors encoding either HA-tagged wild-type (lanes 1–3) or the S33A/S37A mutant of  $\beta$ -catenin (0.7  $\mu$ g) and pCMV5 (1.0  $\mu$ g) (lanes 1 and 3) or either wild-type or mutant  $\beta$ -catenin together with an expression vector encoding the constitutively active FLAG-tagged IKK $\alpha$  (1.0  $\mu$ g) (lanes 2 and 4) or FLAG-tagged IKK $\beta$  (1.0  $\mu$ g) (lanes 3 and 6). Whole cell extracts were prepared and subjected to Western blot analysis with the anti-HA monoclonal antibody 12CA5 to detect the HA-tagged  $\beta$ -catenin (lanes 1–6, top panel) or the M2 monoclonal antibody directed against the FLAG-epitope to detect FLAG-tagged IKK $\alpha$  and IKK $\beta$  (lanes 1–6, lower panel). C, the TOPFLASH reporter was cotransfected with expression vectors encoding LEF-1 and  $\beta$ -catenin and the I $\kappa$ B $\alpha$  super-repressor (0.1, 0.2 and 0.5  $\mu$ g) vectors. In addition, the NF- $\kappa$ B luciferase reporter (100 ng) was transfected with expression vectors encoding either the constitutively active IKK $\beta$  SS/EE or IKK $\alpha$  SS/EE and the I $\kappa$ B $\alpha$  super-repressor. All COS cell transfections contained 0.5  $\mu$ g of an RSV- $\beta$ -galactosidase expression vector, and the DNA amounts were standardized with a pCMV5 vector. Extracts were prepared 18 h after transfection, and the normalized luciferase activity was determined by correcting for differences in  $\beta$ -galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is shown.

would affect the protein levels of a  $\beta$ -catenin mutant in which serine residues 33 and 37 were changed to alanine to result in increased  $\beta$ -catenin protein levels (15–17). Expression vectors encoding either the hemagglutinin-tagged wild-type or S33A/S37A mutant  $\beta$ -catenin were transfected into COS cells either alone or in the presence of either the constitutively active FLAG-tagged IKK $\alpha$  or IKK $\beta$ . Whole cell extracts were prepared from the transfected COS cells and analyzed by Western blot analysis using the 12CA5 and M2 monoclonal antibodies directed against the hemagglutinin and FLAG epitopes, respectively (Fig. 3B). IKK $\alpha$  expression increased the level of the epitope-tagged  $\beta$ -catenin protein (Fig. 3B, lane 2), while IKK $\beta$  reduced the amount of the epitope-tagged  $\beta$ -catenin protein (Fig. 3B, lane 3). In contrast, IKK $\alpha$  did not alter the level of the S33A/S37A  $\beta$ -catenin mutant (Fig. 3B, lane 5), while IKK $\beta$  reduced the level of this protein (Fig. 3B, lane 6). Transfection assays with the TOPFLASH reporter indicated that IKK $\alpha$  increased gene expression in the presence of the wild-type but not the mutant  $\beta$ -catenin, while IKK $\beta$  reduced gene expression in the presence of both of these  $\beta$ -catenin proteins (data not shown). These results suggest that IKK $\alpha$  either directly or indirectly may lead to increased levels of  $\beta$ -catenin to increase TOPFLASH activity, while IKK $\beta$  may reduce the levels of  $\beta$ -catenin to decrease  $\beta$ -catenin activity. The failure of IKK $\alpha$  to further increase the protein levels of the mutant  $\beta$ -catenin suggests that the structure of the amino terminus of  $\beta$ -catenin may be important in this process.

It was important to determine whether activation of the NF- $\kappa$ B pathway may be involved in the increased TOPFLASH activity seen in the presence of  $\beta$ -catenin and LEF-1. The TOPFLASH reporter was transfected with expression vectors encoding  $\beta$ -catenin, LEF-1, and the I $\kappa$ B $\alpha$  super-repressor (I $\kappa$ B $\alpha$  SS/AA) (Fig. 3C). The I $\kappa$ B $\alpha$  super-repressor protein, which contains substitutions of serine residues 32 and 36 with ala-

nine, cannot be phosphorylated by IKK, and its resistance to degradation prevents the nuclear translocation of the NF- $\kappa$ B proteins in response to activators of this pathway (27). The transfection of the I $\kappa$ B $\alpha$  super-repressor did not alter activation of the TOPFLASH reporter in the presence of  $\beta$ -catenin and LEF-1 expression vectors, while it completely abolished the activity of an NF- $\kappa$ B reporter (Fig. 3C). These results suggest that NF- $\kappa$ B activation does not appear to be involved in the activation of TOPFLASH activity by  $\beta$ -catenin and LEF-1.

**The Amino Terminus of  $\beta$ -Catenin Is Critical for IKK $\alpha$  but Not IKK $\beta$  Modulation of Gene Expression**—Next we addressed whether the same or different domains in  $\beta$ -catenin were required for regulation by IKK $\alpha$  and IKK $\beta$ . The amino terminus of  $\beta$ -catenin is phosphorylated by GSK-3 $\beta$  leading to  $\beta$ -catenin degradation (50–52). Amino-terminal deletion mutants of  $\beta$ -catenin are very stable because they lack sequences that are involved in APC-mediated degradation (50–54). Furthermore, our results suggested that the amino terminus of  $\beta$ -catenin may be involved in IKK $\alpha$ -mediated regulation. To determine whether the amino terminus of  $\beta$ -catenin was critical for mediating the effects of IKK $\alpha$  and IKK $\beta$ , transfection of increasing amounts of a  $\beta$ -catenin expression vector deleted of its first 129 amino acids was transfected into COS cells along with LEF-1. There was increased TOPFLASH activity seen with this mutant similar to the results seen with wild-type  $\beta$ -catenin (Fig. 4). The expression of the constitutively active IKK $\beta$  protein reduced activation of TOPFLASH reporter when transfected with this  $\beta$ -catenin mutant. In contrast, the expression of the constitutively active IKK $\alpha$  protein did not alter the ability of the amino-terminal deletion of  $\beta$ -catenin to activate the TOPFLASH reporter (Fig. 4). These results suggest that the decreased  $\beta$ -catenin transactivation observed with IKK $\beta$  is not dependent on the amino terminus of  $\beta$ -catenin, while IKK $\alpha$  requires the presence of this domain to stimulate  $\beta$ -catenin transcriptional activity.

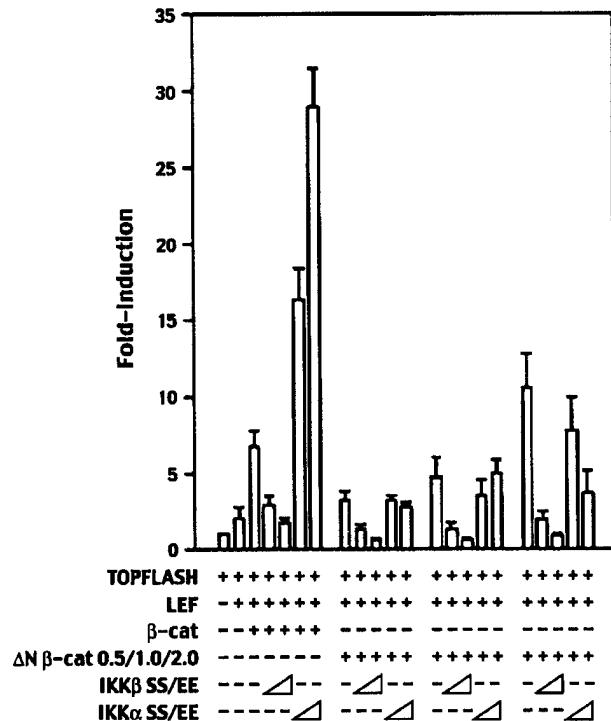


FIG. 4. Differential effects of IKK $\beta$  and IKK $\alpha$  on transactivation of an amino-terminal truncated  $\beta$ -catenin. COS cells were cotransfected with the indicated plasmids including the TOPFLASH reporter, LEF-1, and either wild-type  $\beta$ -catenin (group 1) or an amino-terminal deletion of the first 129 amino acids of  $\beta$ -catenin (groups 2–4). The construct encoding the amino-terminal-deleted  $\beta$ -catenin was cotransfected at concentrations of 0.5 (group 2), 1.0 (group 3), and 2.0  $\mu$ g (group 4) together with the constitutively active kinases IKK $\beta$  SS/EE (0.5 and 1.0  $\mu$ g) and IKK $\alpha$  SS/EE (0.5 and 1.0  $\mu$ g). An RSV- $\beta$ -galactosidase expression vector was added to each transfection, and DNA quantities were standardized by addition of a pCMV5 expression vector. After 18 h, the cells were collected and luciferase activity was determined and normalized to correct for differences in  $\beta$ -galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is presented.

**$\beta$ -Catenin Interacts with IKK $\alpha$  and IKK $\beta$** —To address whether the effects of IKK $\alpha$  and IKK $\beta$  on  $\beta$ -catenin-dependent gene expression may be mediated by direct interactions with  $\beta$ -catenin, we performed coimmunoprecipitation experiments of  $\beta$ -catenin and the IKK proteins using cytoplasmic extracts prepared from the SW480 colon cancer cell line. SW480 cells express a truncated APC gene product and result in enhanced levels of  $\beta$ -catenin. This increased level of  $\beta$ -catenin was necessary to detect this protein in Western blot analysis of column fractions that were generated following chromatography (14).

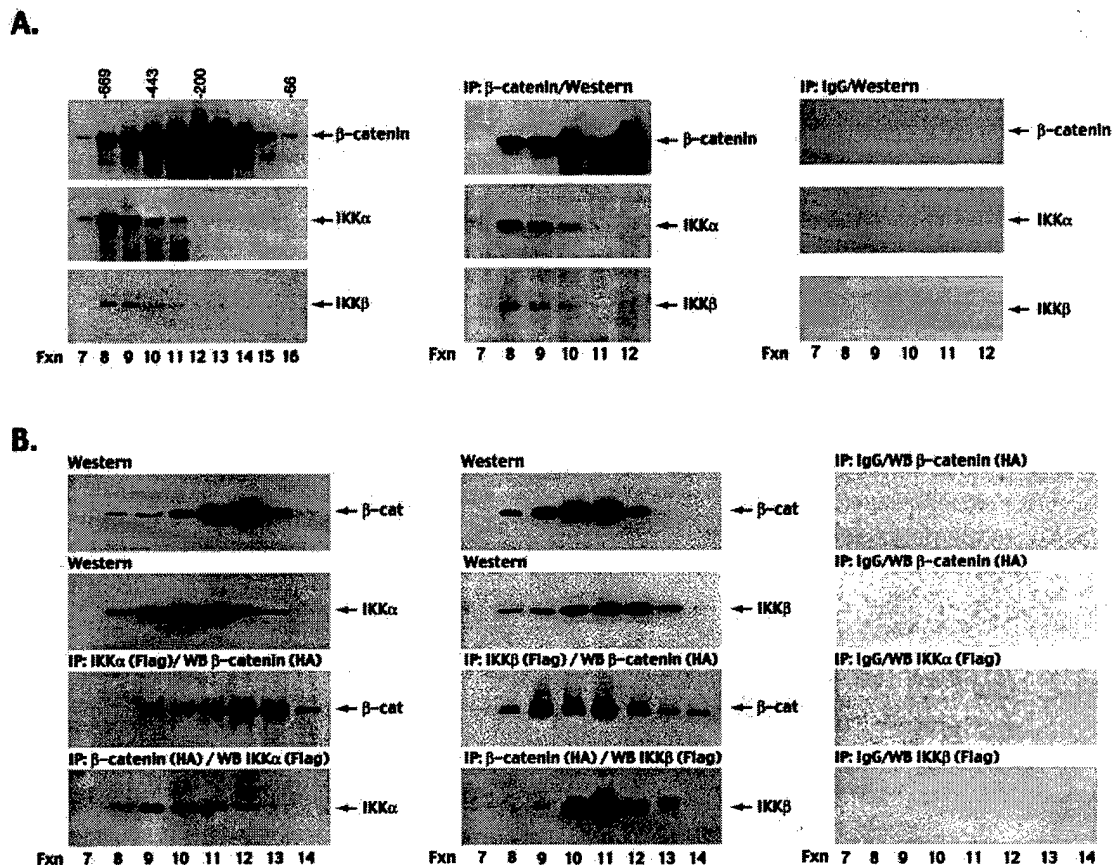
Superdex-200 gel filtration chromatography of the S100 cytoplasmic extract prepared from SW480 cells was utilized to assay interactions between the IKK and  $\beta$ -catenin. Similar chromatographic analysis has previously been used to characterize the high molecular weight IKK complex (55). Western blot analysis of these column fractions indicated that  $\beta$ -catenin was present in a broad peak, including a portion that was present in high molecular weight fractions that also contained IKK $\alpha$  and IKK $\beta$  (Fig. 5A, left panel). Column fractions 7–12, which contained both  $\beta$ -catenin and the IKK proteins, were immunoprecipitated with a monoclonal antibody directed against  $\beta$ -catenin followed by Western blot analysis with either  $\beta$ -catenin, IKK $\alpha$ , or IKK $\beta$  antibodies (Fig. 5A, middle panel). This analysis indicated that  $\beta$ -catenin was associated with IKK $\alpha$  and IKK $\beta$ , while immunoprecipitation of these column

fractions with mouse IgG followed by Western blot analysis demonstrated no association of these proteins (Fig. 5A, right panel). These results suggest that endogenous  $\beta$ -catenin can associate with IKK $\alpha$  and IKK $\beta$ .

Next, we characterized the interactions of IKK $\alpha$  and IKK $\beta$  with  $\beta$ -catenin following cotransfection of COS cells with expression vectors encoding these epitope-tagged proteins (Fig. 5B). First, S100 extracts prepared from these cells were subjected to Superdex-200 chromatography and Western blot analysis. As previously noted, when IKK $\alpha$  and IKK $\beta$  were transfected into COS cells they migrate in a relatively broad peak following Superdex-200 chromatography due to the failure to completely assemble into the high molecular weight IKK complex (49) (Fig. 5B, left and middle panels). Next, immunoprecipitation of column fractions prepared from extracts containing the FLAG-tagged IKK $\alpha$  and HA-tagged  $\beta$ -catenin was performed followed by Western blotting. This analysis indicated that FLAG-tagged IKK $\alpha$  and HA-tagged  $\beta$ -catenin were able to associate (Fig. 5B, left panel). Column fractions of extracts prepared from COS cells cotransfected with FLAG-tagged IKK $\beta$  and HA-tagged  $\beta$ -catenin indicated that both of these proteins were also able to associate (Fig. 5B, middle panel). Western blot analysis of the immunoprecipitated IKK and  $\beta$ -catenin proteins do not strictly overlap. This is likely due to the fact that their elution profiles following chromatography vary, which is consistent with the presence of these proteins in multiple complexes. The column fractions containing HA-tagged  $\beta$ -catenin and either FLAG-tagged IKK $\alpha$  or IKK $\beta$  were also immunoprecipitated with mouse IgG and analyzed by Western blot analysis. This analysis revealed that there were not nonspecific associations of the  $\beta$ -catenin and IKK proteins (Fig. 5B, right panel).

To further characterize the interactions of  $\beta$ -catenin with IKK $\alpha$  and IKK $\beta$ , *in vitro* binding of SW480 cytoplasmic extract with GST proteins fused to different domains of  $\beta$ -catenin was performed. Thus, we could determine the role of different domains of  $\beta$ -catenin including the amino terminus, which regulates protein stability, the armadillo repeats, and the C-terminal transactivation domain in binding the IKK proteins (53). Following the incubation of the SW480 cytoplasmic extract with the GST- $\beta$ -catenin fusion proteins bound to glutathione-Sepharose beads, Western analysis was performed with antibodies directed against either IKK $\alpha$  or IKK $\beta$ . Each of the  $\beta$ -catenin fusion proteins, but not GST alone, was able to interact with IKK $\alpha$  and IKK $\beta$  (Fig. 6B). However, the GST- $\beta$ -catenin fusion proteins extending between amino acid residues 1–400 and 130–400 consistently bound more IKK $\alpha$  and IKK $\beta$  (Fig. 6B). These results suggested that the region of  $\beta$ -catenin containing the first six armadillo repeats was probably critical for interaction with the IKK proteins. The data from the GST-pull down assays in conjunction with coimmunoprecipitation data of both endogenous and transfected proteins demonstrate that the IKK proteins and  $\beta$ -catenin can interact under a variety of different conditions.

**IKK $\alpha$  and IKK $\beta$  Phosphorylate  $\beta$ -Catenin**—Next we addressed whether IKK could phosphorylate the amino terminus of  $\beta$ -catenin and whether stimulation of IKK activity could result in increased  $\beta$ -catenin phosphorylation in *in vitro* kinase assays. The amino terminus of  $\beta$ -catenin has been demonstrated to be a target for GSK-3 $\beta$  phosphorylation (9), while serine residues 32 and 36 in the amino terminus of GST-I $\kappa$ B $\alpha$  are the target for IKK phosphorylation (29–33). HeLa cells were either untreated, treated with TNF $\alpha$ , or transfected with an expression vector encoding NIK (56, 57) to induce IKK kinase activity. The IKK complex was immunoprecipitated from extracts prepared from these cells and assayed for its



**FIG. 5. Association of endogenous and transfected IKK $\alpha$ , IKK $\beta$  and  $\beta$ -catenin proteins.** A, S100 extract was prepared from  $10^8$  SW480 cells and fractionated on a Superdex-200 gel filtration column. Equal volumes (40  $\mu$ l) from each fraction were immunoblotted to monitor distribution of  $\beta$ -catenin, IKK $\alpha$ , and IKK $\beta$  (left panel). The mobility of the different protein markers on the Superdex-200 column are indicated as are the column fraction numbers. For association studies, equal volumes (200  $\mu$ l) from column fractions 7–12, which contained the  $\beta$ -catenin and the IKK proteins, were incubated overnight with monoclonal antibodies directed against  $\beta$ -catenin (Transduction Laboratories). Western blot analysis was then performed on these immunoprecipitates using polyclonal goat antibody to detect  $\beta$ -catenin or rabbit polyclonal antibodies to detect IKK $\alpha$  and IKK $\beta$  (middle panel). These column fractions were also immunoprecipitated with mouse IgG followed by Western blot analysis with polyclonal antibodies directed against either  $\beta$ -catenin, IKK $\alpha$ , or IKK $\beta$  (right panel). B, COS cells were transfected using LipofectAMINE Plus with expression vectors encoding HA-tagged  $\beta$ -catenin and either FLAG-tagged IKK $\alpha$  or FLAG-tagged IKK $\beta$ . Cells were harvested 18 h after transfection, and S100 extracts were prepared and fractionated on a Superdex-200 column. Equal column volumes (40  $\mu$ l) were subjected to Western blot analysis using 12CA5 antibody to detect HA-tagged  $\beta$ -catenin (left and middle panels) or the M2 monoclonal antibody to detect FLAG-tagged IKK $\alpha$  (left panel) or FLAG-tagged IKK $\beta$  (middle panel). Immunoprecipitation of column fractions prepared from FLAG-tagged IKK $\alpha$  and HA-tagged  $\beta$ -catenin (left panel) or FLAG-tagged IKK $\beta$  and HA-tagged  $\beta$ -catenin (middle panel) transfected cells was performed using either the 12CA5 or M2 monoclonal antibodies followed by Western blot analysis with the antibody that was not used in the immunoprecipitation. Column fractions from extracts containing either FLAG-tagged IKK $\alpha$  and HA-tagged  $\beta$ -catenin (right panel, first and third gels) or FLAG-tagged IKK $\beta$  and HA-tagged  $\beta$ -catenin (right panel, second and fourth gels) were immunoprecipitated with mouse IgG followed by Western blot analysis with either 12CA5 (HA) or M2 (FLAG) antibodies as indicated.

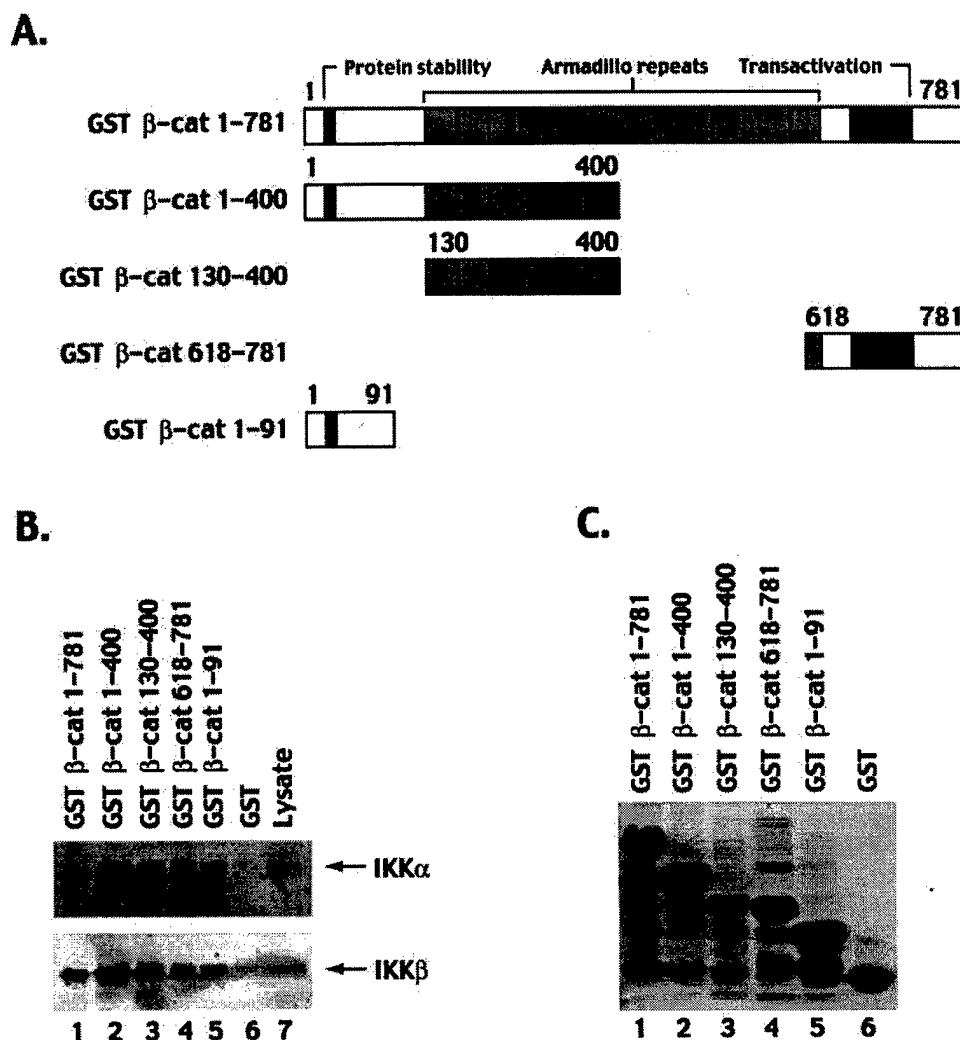
ability to phosphorylate either GST- $\beta$ -cat(1–91), GST-I $\kappa$ B $\alpha$ -(1–54), or GST-I $\kappa$ B $\alpha$  (SS/AA)-(1–54). IKK activity was induced by treatment with either TNF $\alpha$  or NIK and increased the phosphorylation of  $\beta$ -catenin (Fig. 7A, lanes 1–3) and I $\kappa$ B $\alpha$  (Fig. 7A, lanes 4–6), but not the I $\kappa$ B $\alpha$  mutant in which serine residues 32 and 36 were changed to alanine (Fig. 7A, lanes 7–9).

Recombinant baculovirus-produced IKK $\alpha$  and IKK $\beta$  were also tested in *in vitro* kinase assays using GST fusions with  $\beta$ -catenin or I $\kappa$ B $\alpha$ . Both IKK $\alpha$  and IKK $\beta$  also phosphorylated the amino terminus of  $\beta$ -catenin and I $\kappa$ B $\alpha$ , but not the I $\kappa$ B $\alpha$  mutant (Fig. 7B). COS cells were next transfected with either epitope-tagged wild-type or mutant IKK $\alpha$  and IKK $\beta$ , and following immunoprecipitation with the M2 monoclonal antibody these kinases were assayed using *in vitro* kinase assays with  $\beta$ -catenin and I $\kappa$ B $\alpha$  as substrates (48). Wild-type IKK $\alpha$  and IKK $\beta$ , but not the kinase-defective mutants, were able to phosphorylate  $\beta$ -catenin and I $\kappa$ B $\alpha$  (Fig. 7C).

Finally, we addressed whether IKK $\alpha$  and IKK $\beta$  could also phosphorylate additional regions in  $\beta$ -catenin other than its

amino terminus (Fig. 7D). Both kinases phosphorylated GST fusion proteins containing various portions of  $\beta$ -catenin (Fig. 7D, lanes 2–5). These GST fusions contained either the amino terminus of  $\beta$ -catenin, an amino-terminal-deleted form of  $\beta$ -catenin or full-length  $\beta$ -catenin (Fig. 7D). Similar results were obtained using IKK $\alpha$  and IKK $\beta$  preparations produced by baculovirus expression (data not shown). These results indicate that both IKK $\alpha$  and IKK $\beta$  phosphorylate multiple regions of  $\beta$ -catenin.

**Stoichiometry of IKK Phosphorylation of I $\kappa$ B $\alpha$  and  $\beta$ -Catenin**—Next we compared the ability of IKK $\alpha$  and IKK $\beta$  to phosphorylate GST-I $\kappa$ B $\alpha$ -(1–54) and GST- $\beta$ -cat(1–91) substrates. In these *in vitro* kinase assays, we analyzed the phosphorylation of each of these substrates at specific points over a 120-min time course utilizing 0.01 mM, 0.1 mM, and 1.0 mM of cold ATP and 15  $\mu$ Ci of [ $\gamma$ - $^{32}$ ]ATP. Following SDS-PAGE and autoradiography (Figs. 8A and 8B, top panels), the  $^{32}$ P-incorporation into the  $\beta$ -catenin and I $\kappa$ B $\alpha$  substrates was determined, and the number of the moles of phosphate incorporated per mole of



**FIG. 6. In vitro interaction of  $\beta$ -catenin with endogenous IKK $\alpha$  and IKK $\beta$ .** A, a schematic representation of the GST  $\beta$ -catenin fusion proteins that were used to analyze interactions with SW480 extract is shown. B, GST-fusion proteins with  $\beta$ -catenin were bound to glutathione-Sepharose beads (lanes 1–6), and after overnight incubation with SW480 cell extract, the glutathione beads were extensively washed and Western blot analysis was performed using rabbit polyclonal antibodies directed against either IKK $\alpha$  or IKK $\beta$  as indicated. 10% of the SW480 lysate alone is shown in lane 7. C, the GST-fusion proteins were analyzed by Coomassie Blue staining.

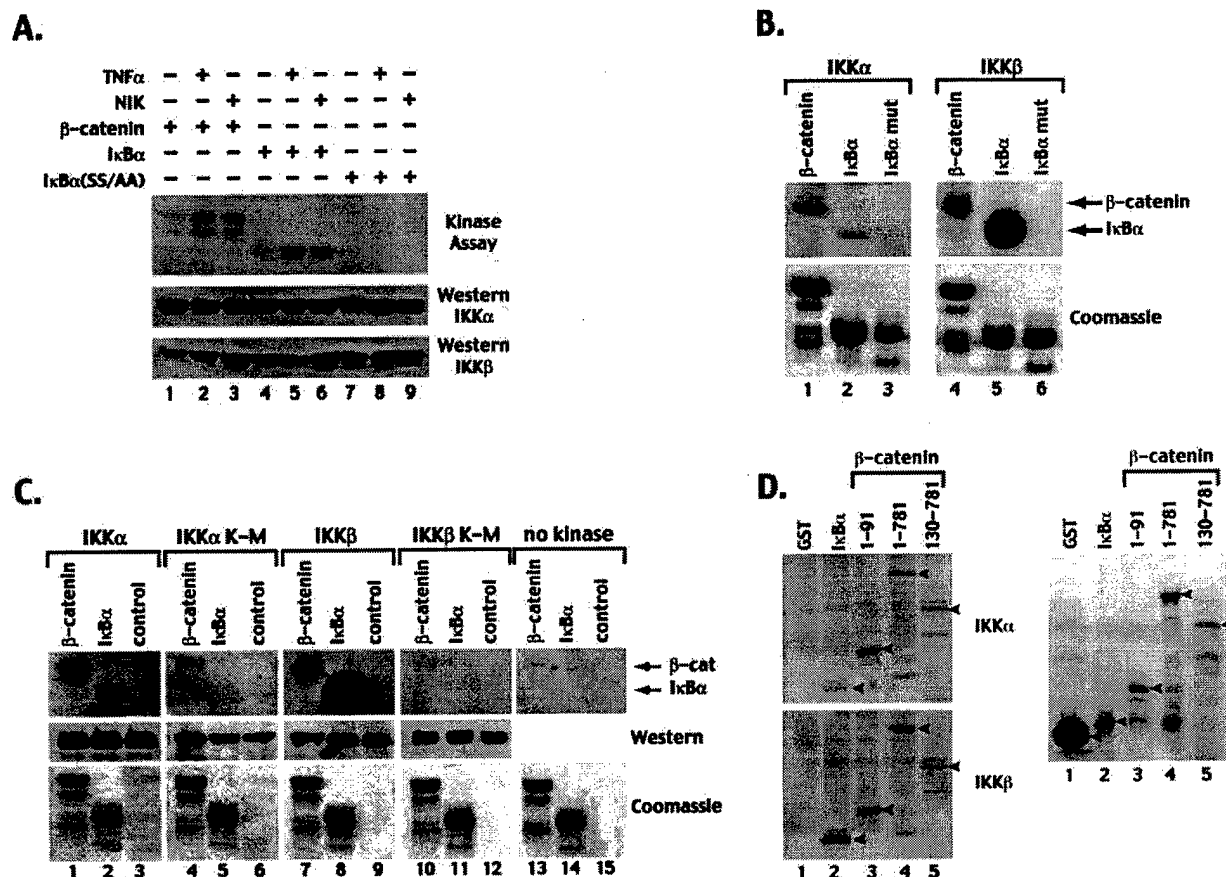
substrate was calculated (Fig. 8, A and B, lower panels).

Kinase assays performed with IKK $\alpha$  using 1 mM of cold ATP resulted in  $\sim 0.05$  mol of phosphate/mol of protein incorporated into the amino terminus of  $\beta$ -catenin as compared with 0.09 mol of phosphate/mol of protein incorporated into the amino terminus of  $I\kappa B\alpha$  after a 120-min reaction (Fig. 8A). Kinase assays performed with IKK $\beta$  using 1 mM of cold ATP demonstrated that there was 1.4 mol of phosphate/mol of protein incorporated into the amino terminus of  $\beta$ -catenin and 0.5 mol of phosphate/mol of protein incorporated in the amino terminus of  $I\kappa B\alpha$  after 120 min (Fig. 8B). It is interesting to note that the phosphorylation of the  $\beta$ -catenin by IKK $\beta$  may be biphasic in contrast to its phosphorylation of  $I\kappa B\alpha$  (Fig. 8B). Similar phosphate incorporation into these substrates was found using both baculovirus-produced and COS-transfected IKK $\alpha$  and IKK $\beta$  proteins (data not shown). In agreement with previous studies, this analysis indicates that IKK $\alpha$  is a much weaker kinase than is IKK $\beta$  in phosphorylating  $I\kappa B\alpha$  (58) and  $\beta$ -catenin. These results indicate that the IKK proteins result in relatively similar incorporation of phosphate into the amino terminus of  $\beta$ -catenin and  $I\kappa B\alpha$ , although there are differences in the kinetics of this process.

#### DISCUSSION

In this study, we present data that IKK $\alpha$  and IKK $\beta$  can modulate  $\beta$ -catenin function. First, we observed the differential localization of  $\beta$ -catenin in mouse embryo fibroblasts derived from IKK $\alpha$ - and IKK $\beta$ -deficient cells. Second, the transcriptional activity of  $\beta$ -catenin was higher in IKK $\beta$ <sup>-/-</sup> cells as compared with IKK $\alpha$ <sup>-/-</sup> cells. Third, IKK $\beta$  decreased  $\beta$ -catenin-dependent gene expression similar to the effects seen with GSK-3 $\beta$ , while IKK $\alpha$  increased this activity. Fourth, we found that IKK $\alpha$  expression in COS cells increased the amount of  $\beta$ -catenin, while IKK $\beta$  expression reduced the amount of  $\beta$ -catenin. Finally, we demonstrated that IKK $\alpha$  and IKK $\beta$  interacted with and were able to phosphorylate  $\beta$ -catenin. Experiments are underway to map the sites in  $\beta$ -catenin that are phosphorylated by IKK $\alpha$  and IKK $\beta$  in order to determine whether phosphorylation alters  $\beta$ -catenin function. Our preliminary results suggest that IKK $\alpha$  phosphorylates different residues in the amino terminus of  $\beta$ -catenin than serine residues 33 and 37 that are phosphorylated by GSK-3 $\beta$ .

Studies with an amino-terminal deletion of  $\beta$ -catenin indicated that IKK $\alpha$  requires this region to increase  $\beta$ -catenin-de-



**FIG. 7. IKK $\alpha$  and IKK $\beta$  phosphorylate  $\beta$ -catenin and I $\kappa$ B $\alpha$ .** **A**, HeLa cells were either untreated, treated with TNF $\alpha$  (20 ng/ml) for 10 min, or transfected with a pCMV5 expression vector encoding NIK. Extracts were immunoprecipitated with a polyclonal antibody directed against IKK $\alpha$  and IKK $\beta$  and *in vitro* kinase assays were performed with GST fusions with  $\beta$ -catenin-(1-91), I $\kappa$ B $\alpha$ -(1-54) or I $\kappa$ B $\alpha$  SS/AA-(1-54) followed by autoradiography. **B**, recombinant baculovirus-expressed IKK $\alpha$  and IKK $\beta$  proteins were purified as described. *In vitro* kinase assays were performed using IKK $\alpha$  and IKK $\beta$  and GST- $\beta$ -cat-(1-91) (lanes 1 and 4) and GST-I $\kappa$ B $\alpha$ -(1-54) (lanes 2 and 5) as substrates (upper panel). A GST-I $\kappa$ B $\alpha$  protein in which serine residues 32 and 36 were changed to alanine (lanes 3 and 6) was also assayed. The GST fusion proteins used in the *in vitro* kinase assay were monitored by Coomassie Blue staining (lower panel). **C**, COS cells were transfected with expression vectors encoding either FLAG-tagged wild-type or mutant IKK $\alpha$  or IKK $\beta$  kinases or mock transfected (no kinase). After 30 h, the cells were collected, and cellular extract was immunoprecipitated with the M2 monoclonal antibody directed against FLAG-epitope. The upper panel demonstrates *in vitro* phosphorylation of either GST- $\beta$ -catenin (lanes 1, 4, 7, 10, and 13), GST-I $\kappa$ B $\alpha$  (lanes 2, 5, 8, 11, and 14) or no added substrate (lanes 3, 6, 9, 12, and 15) by the indicated kinases. Expression of the transfected IKK constructs was analyzed by Western blot analysis (middle panel). Immunoprecipitation of extracts from mock-transfected cells were also analyzed in *in vitro* kinase assays (lanes 13-15). The amount of GST- $\beta$ -catenin and GST-I $\kappa$ B $\alpha$  substrates used in these assays was monitored by Coomassie Blue staining (lower panel). **D**, FLAG-tagged IKK $\alpha$  (top panel) and IKK $\beta$  (bottom panel) immunoprecipitated from COS cell extracts were used in *in vitro* kinase assays with GST alone (lane 1) or GST fusions containing I $\kappa$ B $\alpha$ -(1-54) (lane 2),  $\beta$ -catenin-(1-91) (lane 3),  $\beta$ -catenin-(1-781) (lane 4), or  $\beta$ -catenin-(130-781) (lane 5). SDS-PAGE and autoradiography were then performed. A Coomassie-stained gel of the substrates used in the *in vitro* kinase assays is shown in the right panel.

pendent gene expression, while the effects of IKK $\beta$  on  $\beta$ -catenin activity are not dependent on this region. These results and the finding that IKK $\alpha$  is not able to increase the protein levels of an amino-terminal  $\beta$ -catenin mutant suggest that the amino terminus of  $\beta$ -catenin is likely involved in IKK $\alpha$  regulation. Thus, IKK $\alpha$  and IKK $\beta$  likely have effects on different domains of  $\beta$ -catenin to alter its role on gene expression. It is unclear whether differences in the kinase activity of IKK $\alpha$  and IKK $\beta$  are involved in their differential effects on  $\beta$ -catenin-dependent gene expression or whether other effects such as differential binding to specific pools of  $\beta$ -catenin may be involved. Finally, additional mechanisms such as IKK effects on  $\beta$ -catenin protein stability and/or nuclear import or export are possible. Multiple factors including Wnt signaling, the TCF/LEF proteins (19, 52, 53), and APC (59) affect the cellular localization of  $\beta$ -catenin, which lacks a canonical nuclear localization signal. Although we demonstrate that the IKK proteins interact with  $\beta$ -catenin, it is possible that IKK interaction with other com-

ponents of the Wnt pathway such as APC may also be involved in regulating  $\beta$ -catenin function.

Both IKK $\alpha$  and IKK $\beta$  can form heterodimers and homodimers, and dimerization of these kinases is essential for their activity (33, 47, 60, 61). However, previous data has suggested that there is no synergy between IKK $\alpha$  and IKK $\beta$  in regulating their kinase activity (58). Given the wide disparity in their kinase activity, they may have other cellular targets in addition to I $\kappa$ B (58). The ability of these kinases to potentially associate with as yet unidentified cellular factors may alter their substrate specificity. Gene disruption studies indicate that IKK $\beta$  rather than IKK $\alpha$  is the critical kinase involved in the activation of the NF- $\kappa$ B pathway in response to treatment with either TNF $\alpha$  or IL-1 $\beta$  (37-39). The predominant cytoplasmic localization of IKK $\beta$  probably reflects the major role of this kinase in the phosphorylation of the I $\kappa$ B proteins that are localized in the cytoplasm bound to the RelA/p65 NF- $\kappa$ B protein (37-39). The results of our immunofluorescence studies



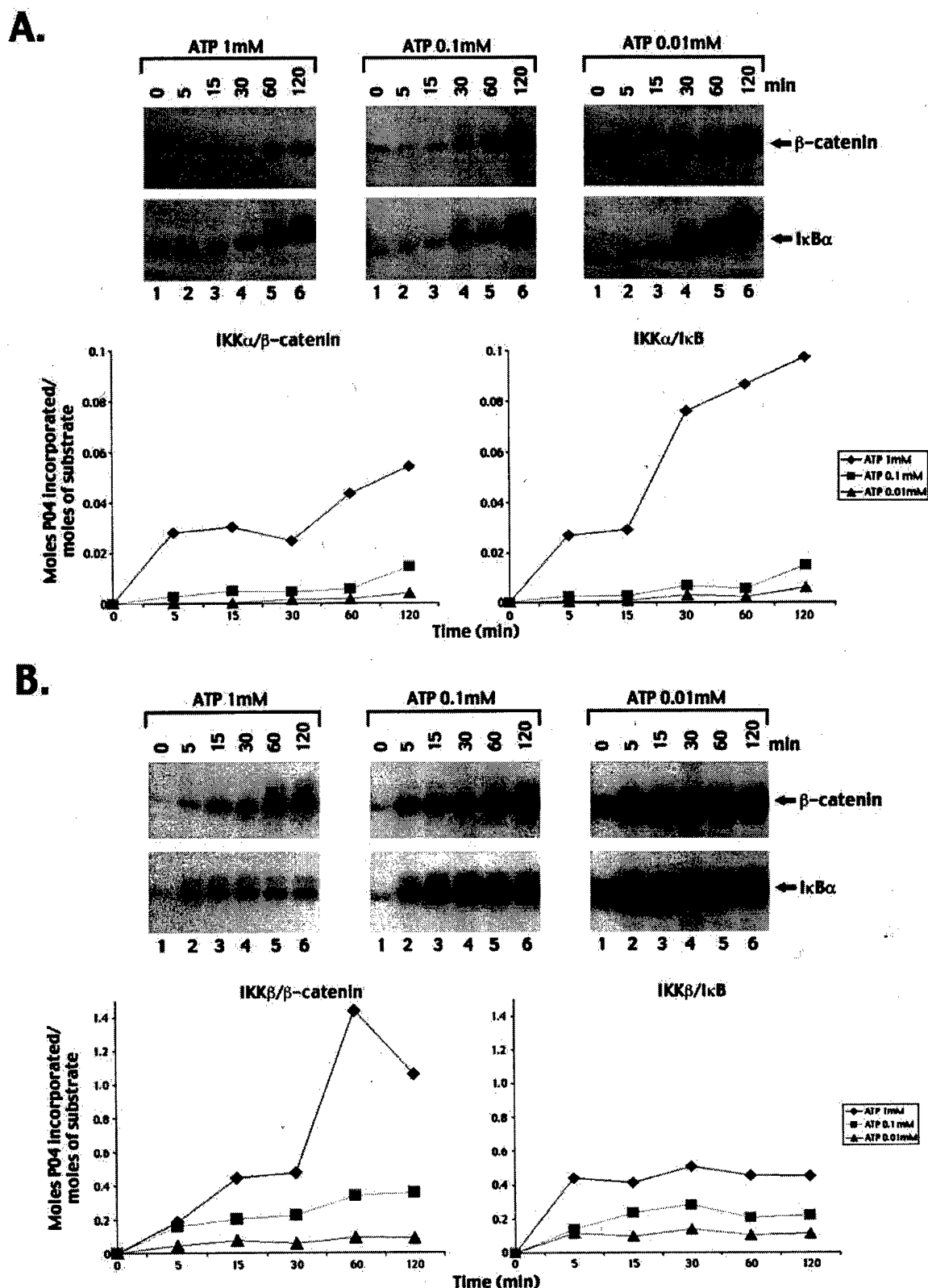


FIG. 8. Stoichiometry of phosphate incorporation into the amino termini of  $I\kappa B\alpha$  and  $\beta$ -catenin by  $IKK\alpha$  and  $IKK\beta$ . **A**, FLAG-tagged  $IKK\alpha$  and **B**,  $IKK\beta$  were immunoprecipitated from COS extracts with the M2 monoclonal antibody and incubated with 2  $\mu$ g of GST fusions containing either  $\beta$ -catenin-(1-91) or  $I\kappa B\alpha$ -(1-54) in kinase buffer containing 15  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP with a specific activity of 6000 Ci/mM and cold ATP at concentrations of 1 mM, 0.1 mM, and 0.01 mM. In vitro kinase reactions were performed for 0, 5, 15, 30, 60, and 120 min at 30 °C, and the samples were subjected to SDS-PAGE and autoradiography (**A** and **B**, top panels). Incorporation of  $^{32}$ P into these substrates was quantitated by scintillation counting and the moles of phosphate incorporated per mole of substrate was calculated (**A** and **B**, bottom panels).



suggest that IKK $\alpha$  is localized in both the nucleus and cytoplasm of MEFs and may be predominantly nuclear in the absence of IKK $\beta$  in IKK $\beta^{-/-}$  cells. Consistent with these observations, Western blot analysis of extracts prepared from COS cells transfected with expression vectors encoding IKK $\alpha$  and IKK $\beta$  indicate that IKK $\beta$  is predominantly localized in the cytoplasm, while IKK $\alpha$  is present in both the nucleus and the cytoplasm. Additional studies are currently underway to better characterize the cellular localization of IKK $\alpha$ . Whether any of the effects of IKK $\alpha$  on skin and skeletal development may in part be mediated by either IKK $\alpha$  binding and/or phosphorylation of  $\beta$ -catenin remains to be determined.

IKK regulation of  $\beta$ -catenin activity differs from its activation of the NF- $\kappa$ B pathway. Cytokines such as TNF $\alpha$  stimulate IKK phosphorylation of I $\kappa$ B leading to its rapid degradation and the nuclear translocation of NF- $\kappa$ B. TNF $\alpha$  activation of an NF- $\kappa$ B reporter construct is blocked by transfection of an IKK $\beta$  dominant negative mutant (30). Although TNF $\alpha$  treatment of cells results in marked decreases in  $\beta$ -catenin-dependent gene expression, this effect is only partially blocked by an IKK $\beta$  dominant negative mutant. These results suggest that the effects of TNF $\alpha$  on  $\beta$ -catenin-dependent gene expression likely involve additional substrates and/or pathways other than IKK $\beta$  and  $\beta$ -catenin. Although our results support a role for IKK $\alpha$  and IKK $\beta$  on modulating  $\beta$ -catenin activity, the regulation of this pathway is different from that seen with TNF $\alpha$ -induction of IKK to activate the NF- $\kappa$ B pathway.

Several observations are also consistent with the potential for similar factors being involved in the regulation of the Wnt and NF- $\kappa$ B pathways. It has been demonstrated that  $\beta$ -catenin/TCF signaling increases  $\beta$ -TrCP levels by a posttranscriptional mechanism to result in increased degradation of both  $\beta$ -catenin and I $\kappa$ B (45). Thus, changes of  $\beta$ -TrCP levels can result in marked effects on both the  $\beta$ -catenin and NF- $\kappa$ B pathways. Additionally, GSK-3 $\beta$ , which is an important kinase involved in regulating  $\beta$ -catenin levels, has also been implicated in regulating NF- $\kappa$ B activation. Gene disruption studies have indicated that GSK-3 $\beta^{-/-}$  mice have a phenotype similar to IKK $\beta$ -deficient mice developing liver degeneration as a result of increased sensitivity to TNF $\alpha$  stimulation (46). The mechanism by which GSK-3 $\beta$  may alter the NF- $\kappa$ B pathway remains to be determined. In summary, our studies suggest that a common set of cellular factors may be involved in the integration of a variety of cellular signaling processes that regulate the NF- $\kappa$ B and  $\beta$ -catenin pathways.

**Acknowledgments**—We thank Ken Kinzler for providing  $\beta$ -catenin and the TOPFLASH reporter constructs, Rudolf Grosschedl for providing the expression vector encoding LEF-1, Masahiro Aoki for providing additional LEF constructs, and Xiaodong Wang for providing the MEF cells. We also thank Melanie Cobb for helpful suggestions and Sharon Johnson and Alex Herrera for preparation of the manuscript and figures, respectively.

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